

Microbial Production of Volatile Organic Compounds in Soil Microcosms

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ABSTRACT

A study was conducted to evaluate the use of volatile organic microbial metabolites, particularly geosmin and 2-methylisoborneol, as indicators of activity of specific groups of microorganisms (actinomycetes, bacteria, or fungi) in soil. Substrates and selective antibiotics were added to soil microcosms to establish different soil microbial communities; volatile organic compounds (VOCs) in the headspace atmosphere of the microcosms were collected by purge-and-trap methods and analyzed by gas chromatography-mass spectrometry. Treatments differed in total amount of VOCs produced, numbers and kinds of VOCs detected, and in temporal patterns of VOC production. The greatest total amount of VOCs and largest number of different VOCs were produced in the treatment with greatest actinomycetal and bacterial activity, followed by the treatment dominated by fungal activity. Geosmin was occasionally detected in small amounts but only in microcosms with substantial activity of all three groups of microorganisms (actinomycetes, bacteria, and fungi). 2-Methylisoborneol was regularly detected in significant amounts but only in the treatment dominated by actinomycetes and bacteria. Terpenes were the VOCs produced in greatest quantity in the treatment with the most fungal activity. Results indicate that VOCs from soil may provide information as to the nature of the soil microbial community; however, relating the structure of the microbial community to soil VOC emissions is complicated because VOCs present in natural soils may be derived from a variety of sources and their production is strongly influenced by environmental conditions.

TO BETTER UNDERSTAND the structure and functioning of microbial communities in soil and their response to soil management practices or perturbations, it is necessary to differentiate the activities of the major groups of soil microorganisms, e.g. fungi, bacteria, and actinomycetes. Two techniques have been used to separate the activities of fungi and bacteria in soil. These methods are designed to estimate the relative contributions of fungi and bacteria to soil respiration through the use of selective microbial biocides (Anderson and Domsch, 1973) or selective metabolizable dyes (Stamatiadis et al., 1990). However, application of selective microbial biocides to soil may cause changes in microbial community structure and allow indirect or nontarget effects to occur (Ingham and Coleman, 1984). The direct microscopic counting procedure involved in enumerating selectively stained microorganisms is subject to a high degree of observer-associated variability and, for this reason, comparisons of values obtained by different labs are tenuous (Stahl et al., 1995). Therefore, additional techniques to aid in the differentiation of activities of groups of microbes in soil would be very useful. Soil is a major source of VOCs, most of which are formed as the result of the metabolic activity of microorganisms (Stotzky and Schenck, 1976). Fungi, bacteria, and actinomycetes in

soil produce a wide variety of VOCs including alcohols, aliphatic and aromatic aldehydes, esters, amines, methylated halogens, terpenoids, and volatile fatty acids (Linton and Wright, 1993). Studies have shown that different species of microorganisms produce different volatile organic metabolites (Grametbauer et al., 1988; Borjesson et al., 1992). Thus, the amount and kinds of organic volatiles produced in a soil is controlled by, among other things, the structure and activity of the microbial community.

Geosmin and 2-methylisoborneol are VOCs produced as metabolites of microorganisms and are important contributors to the earthy/musty odor of soil (Gerber and Lechevalier, 1965; Buttery and Garibaldi, 1976). Both compounds are produced by certain actinomycetes (Gerber and Lechevalier, 1965; Gerber, 1979) and fungi (Karahadian et al., 1985; Mattheis and Roberts, 1992). These two compounds are unique in that they can be detected by the human nose in air at concentrations in the low parts per billion range (Buttery et al., 1969). In a study of purge-and-trap methods for extraction of geosmin and 2-methylisoborneol from soil, Stahl and Parkin (1994) compared two soils and found that the one with highest extractable levels of these two compounds also had the most fungal hyphae and actinomycete filaments, supporting their hypothesis that these compounds may be good indicators of activity of filamentous organisms.

The objectives of this study were to determine if soils inhabited by different communities of microorganisms produce different kinds and amounts of VOCs, and if the VOCs produced reveal information about the structure of the microbial community. To accomplish this goal, substrates and selective antibiotics were added to soil microcosms to establish different soil microbial communities; VOCs produced in the microcosms were collected by purge-and-trap methods and analyzed by gas chromatography-mass spectrometry, with special regard given to geosmin and 2-methylisoborneol. Patterns of VOC production were then related to the structure of the microbial community.

MATERIALS AND METHODS

Soil used in this experiment was collected from the Doolittle Native Prairie Preserve in Story County, Iowa (Kossuth silty clay loam, a Typic Endoaquoll). Approximately 7 kg of soil were returned to the lab, allowed to air dry slightly, and passed through a 4-mm sieve. Two hundred grams of this soil were then placed in 500-mL Erlenmeyer flasks with a 24/40 joint neck to accommodate the VOC sampling apparatus.

Soil in the flasks was amended as follows to produce five treatments. Treatment 1 (control): 1.0 g talc was added and thoroughly mixed into the soil in each of five flasks. Treatment 2: 1.0 g talc and 5.0 g dried, finely ground alfalfa (*Medicago sativa* L.) leaves. Treatment 3: 1.0 g talc and 5.0 g dried, finely ground wheat (*Triticum aestivum* L.) straw. Treatment 4: 0.4 g streptomycin (bactericide) diluted in 1.0 g talc and

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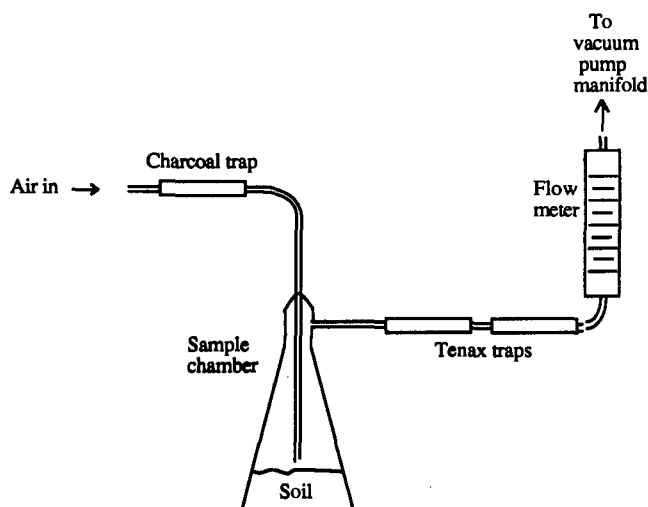


Fig. 1. Diagrammatic representation of purge-and-trap apparatus for collection of volatile organic compounds produced in soil microcosms.

5.0 g dried, finely ground alfalfa leaves. Treatment 5: 0.4 g cycloheximide (fungicide) diluted in 1.0 g talc and 5.0 g dried, finely ground alfalfa leaves. Wheat straw and alfalfa leaves used in this experiment were harvested and air-dried 7 mo prior to use. Talc was used as a diluent for even distribution of antibiotics in soil (Anderson and Domsch, 1978) and the final concentration of antibiotics in Treatments 4 and 5 was 2 g kg^{-1} soil. Sterile distilled water (13.0 mL) was added to all flasks after substrate and antibiotic additions to bring the gravimetric soil water content to 25%. The top of each flask was covered with an aluminum foil cap and the microcosms were incubated at room temperature ($23\text{--}24^\circ\text{C}$). This study was designed as a completely randomized experiment with each of the treatments replicated five times for a total of 25 microcosms.

Every 3 d for the first 18 d of the experiment, and then on Day 24, headspaces of the microcosms were analyzed for both VOC and CO_2 . Soil was sampled for estimation of populations of bacteria, actinomycetes, and fungi every 3 d for the first 12 d of the experiment, and then on Days 18 and 24. To determine the CO_2 concentration in the headspace of the microcosms, a 5.0-mL sample was withdrawn from the flask, through the foil cover, using a gas-tight syringe. Analysis of VOCs was accomplished by purge-and-trap methods as follows (Fig. 1). The foil cover was removed from the top of the flask and quickly replaced with the inner glassware of a vacuum trap (with a 24/40 joint to fit the flask). The VOCs were purged from the microcosms with a flow of air and collected on an in-line trap consisting of silanized glass tubes (11.5 cm by 4 mm i.d.) loosely packed with Tenax TA (a porous polymer sorbent, Alltech Associates, Deerfield, IL)¹ attached directly to the outlet of the apparatus (Stahl and Parkin, 1994). The vacuum line was connected to the outlet end of the trap to create an air flow through the system, flushing VOCs from the microcosm and drawing them into the trap. Air entering the system was passed through an activated charcoal filter to prevent contamination of the sample. Microcosms were purged for 2 h at a flow rate of 25 mL min^{-1} .

¹ Mention of trademark or proprietary product is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product to the exclusion of others that may also be suitable.

Volatile organic compounds were desorbed from Tenax traps using a Supelco thermal tube desorber (Model 850), separated and quantified with a Hewlett Packard gas chromatograph (Model 5890) and mass spectra of compounds were obtained using a Hewlett Packard mass spectrometer (Model 5989). Compounds were desorbed at 250°C for 5 min with a gas flow of 12 mL min^{-1} and carried directly to the column of the gas chromatograph by a heated (170°C) transfer line. The gas chromatograph was equipped with a fused silica (DB-5) megabore column (30 m by 0.552 mm) and a flame ionization detector. The oven temperature program started at an initial temperature of 40°C for 10 min and then increased at a rate of $10^\circ\text{C min}^{-1}$ to a final temperature of 255°C for 15 min. The mass spectrometer was operated in the electron ionization mode with the source temperature at 250°C , ionization energy of 70 eV, and an electron multiplier voltage of 2200. The instrument was autotuned with perfluorotributylamine using mass 69, 219, and 502. The VOCs in the headspace atmospheres of microcosms were identified by computer matching of mass spectra of unknown compounds to known mass spectra of compounds in the Hewlett Packard computerized Wylie/NBS registry of mass spectral data (McLafferty, 1989).

Direct microscopic examination of fungal hyphae and actinomycete filaments in soil was conducted using the membrane filter method (Hanssen et al., 1974), with calcofluor M2R white as stain for slide preparation, and the gridline intersect method (Olson, 1950) for measuring hyphal lengths. For each slide made, 1 g soil (wet weight) was dispersed in 500 mL nanopure filtered water in a blender for 1.5 min. One milliliter of the suspension was passed through a 25-mm-diam. $0.4\text{-}\mu\text{m}$ mesh polycarbonate membrane filter. The material remaining on the filter was then stained for 15 to 20 s with 1 mL of a $2.3 \mu\text{g mL}^{-1}$ aqueous solution of calcofluor white dispensed with a syringe equipped with a $0.2\text{-}\mu\text{m}$ filter. Stain was removed by vacuum followed by rinsing with nanopure filtered water. The stained filter was placed on a glass microscope slide, allowed to air dry for a few seconds to remove excess moisture, mounted with two drops of immersion oil, and covered with a glass cover slip. The number of bacteria cells in a soil was also estimated using direct microscopic methods similar to those used for fungi and actinomycetes except that soil samples were diluted in a buffer solution ($10 \text{ mM Na}_2\text{HPO}_4$) to a final concentration of 1:250 000 and stained with acridine orange (0.01%). Slides were examined with a Nikon Microphot-SA epifluorescent microscope equipped with a high-intensity mercury light source and a Nikon UV-1A filter cube. Observations of fungi and actinomycetes were made using a dry $40\times$ objective, $10\times$ oculars, and $1.5\times$ light path magnifier (total magnification $600\times$). Bacteria were observed using a $100\times$ oil objective, $10\times$ oculars, and a Nikon B-2H filter cube. Twenty-five randomly chosen fields of view were counted on each slide.

RESULTS

Characterization of microbial communities in the five treatments are given in Fig. 2. Numbers of microorganisms remained fairly constant in the control treatment to which no substrates or antibiotics were added. Populations of all three groups examined (actinomycetes, bacteria, and fungi) increased substantially with addition of alfalfa alone (Treatment 2), whereas appending with straw alone (Treatment 3) resulted in much less pronounced increases. In both Treatments 2 and 3, greatest population growth was observed in the fungi. By Day 24, populations of all three groups of microorganisms

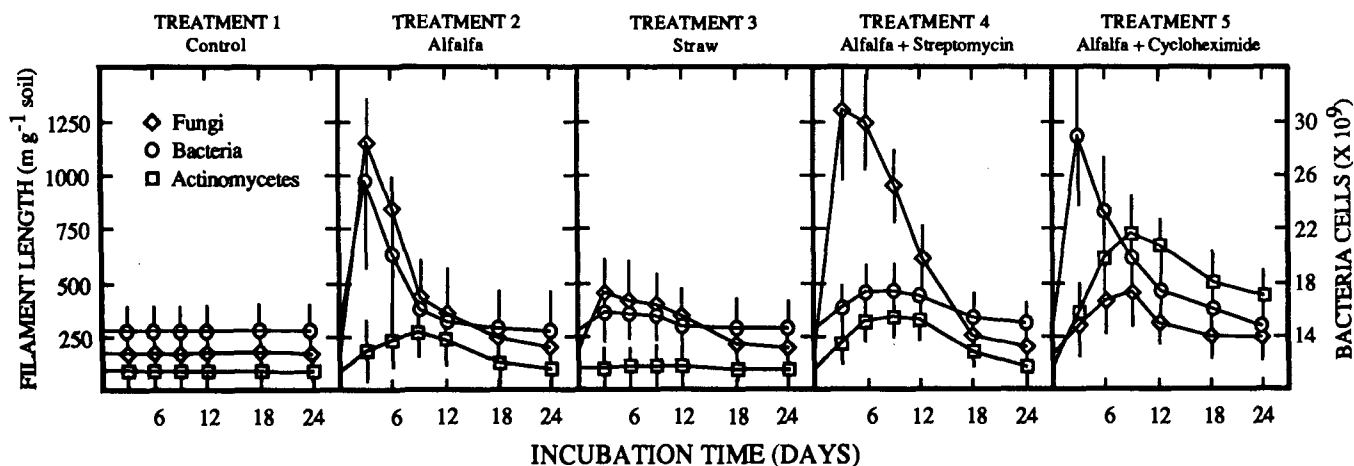


Fig. 2. Populations of actinomycetes, bacteria, and fungi in soil microcosms for the five experimental treatments during the course of the experiment. Each data point represents the mean of five observations. Error bars = ± 1 standard error.

in all five treatments had returned to levels similar to those at the beginning of the experiment. Fungal activity was greatest in microcosms to which alfalfa and streptomycin were added (Treatment 4) but smaller increases in actinomycete and bacteria populations were also observed. Growth of actinomycetes and bacteria was greatest with addition of alfalfa and cycloheximide but a relatively small, slow expansion of the fungal population was also detected. Appending Treatments 4 and 5 with antibiotics did not completely inhibit growth of target organisms but resulted in greater activity of nontarget organisms compared with Treatment 2.

Total microbial activity or respiration, as indicated by headspace CO₂ concentration (Fig. 3), remained constantly low in Treatment 1 and reached highest levels in the treatment with substantial growth of all three groups of microbes (Treatment 2) and the treatment dominated by fungi (Treatment 4). Carbon dioxide production also reached a fairly high level in the treatment dominated by actinomycetes and bacteria (Treatment 5). Highest levels of CO₂ production were always detected within the first 6 d of the experiment; after 24 d, CO₂ production in all treatments had returned to levels close

to those of soil to which no substrate or antibiotics were added.

Composition of the headspace atmosphere of microcosms was different for all five treatments. Representative chromatograms of headspace VOCs for all treatments 12 d into the experiment are given in Fig. 4. Headspace atmospheres differed in total amount of VOCs produced, number and kinds of VOCs detected, and in their temporal patterns of VOC production (Fig. 5 and 6). The greatest total amount of VOCs was produced in microcosms dominated by actinomycete and bacterial activity (Treatment 5) followed, in descending order, by microcosms dominated by fungi (Treatment 4), those in which there was substantial activity of all three groups (Treatment 2), and the treatment to which only straw was added (Treatment 3). In all treatments, highest levels of VOC production always occurred 6 to 12 d after the peak in respiration (Fig. 3). The largest number of different VOCs (22) was detected in Treatment 5 (Table 1), followed by Treatments 4 and 2 with 15 and 12, respectively.

The amounts and kinds of VOCs produced in Treatment 1 remained relatively constant throughout the exper-

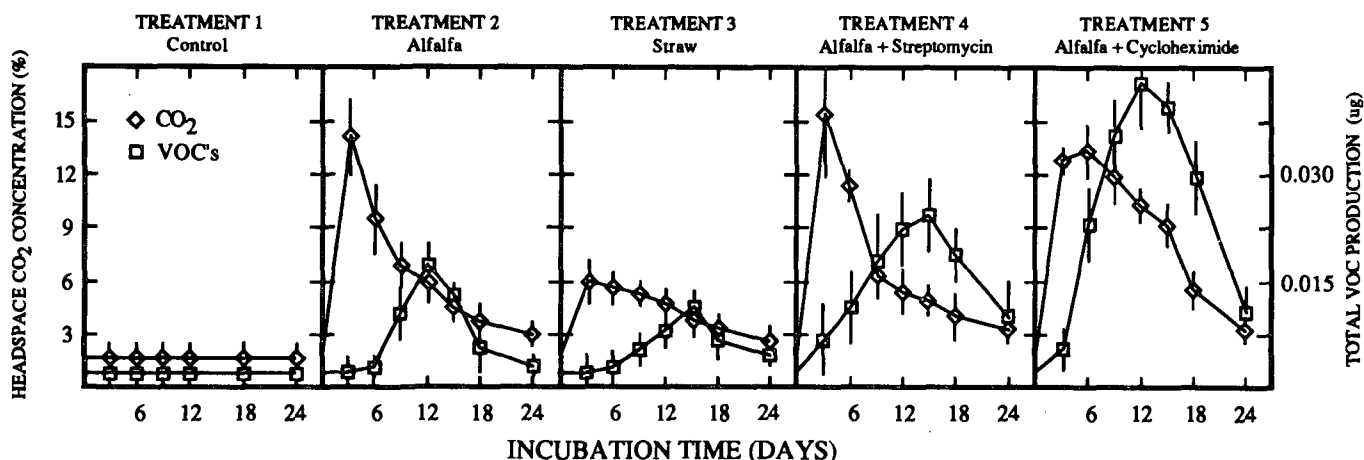


Fig. 3. Carbon dioxide and volatile organic compound production in soil microcosms for the five experimental treatments during the course of the experiment. Each data point represents the mean of five observations. Error bars = ± 1 standard error.

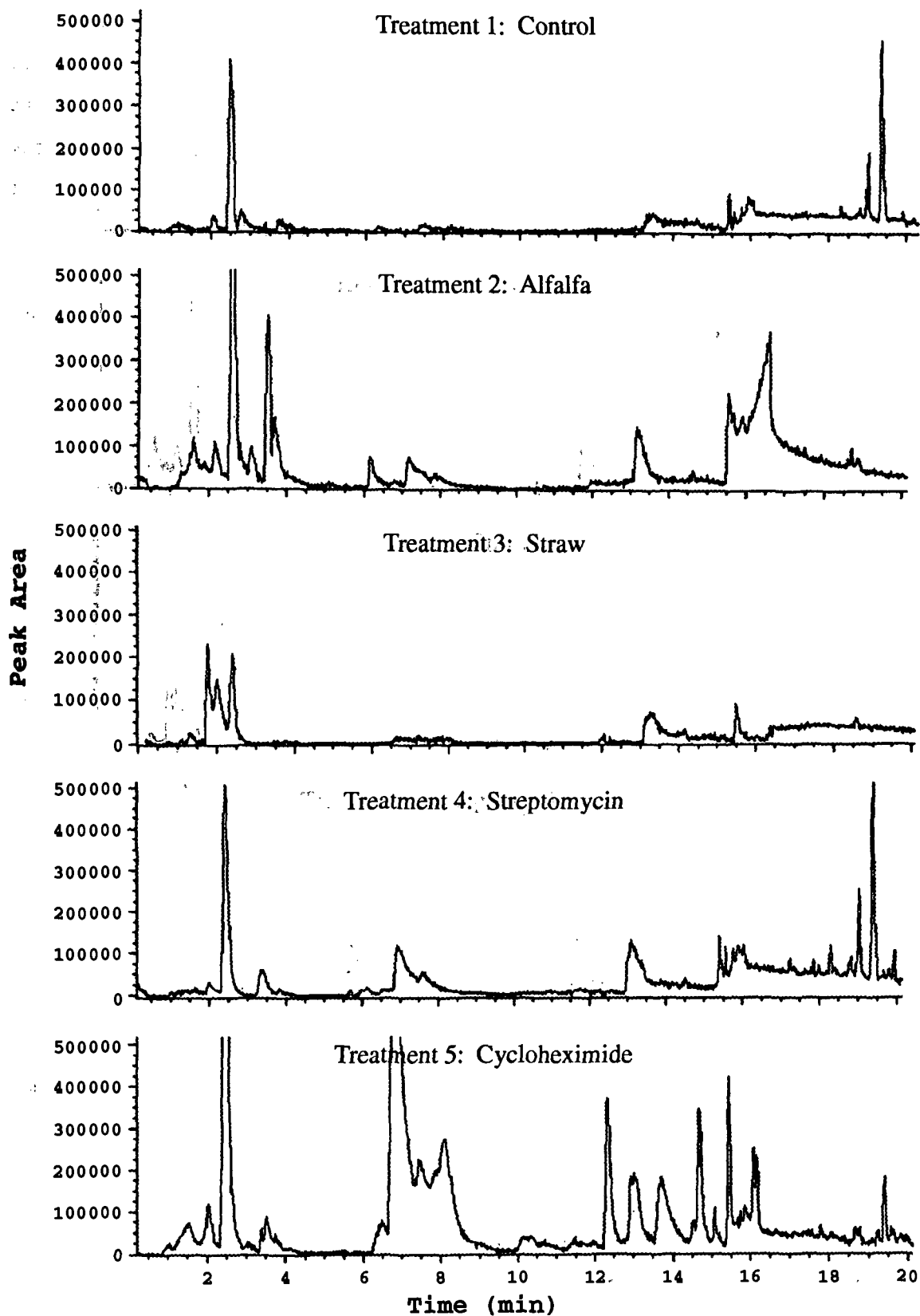


Fig. 4. Representative chromatograms of volatile organic compounds (VOCs) in headspace atmosphere of the five experimental treatments during period of peak VOC production.

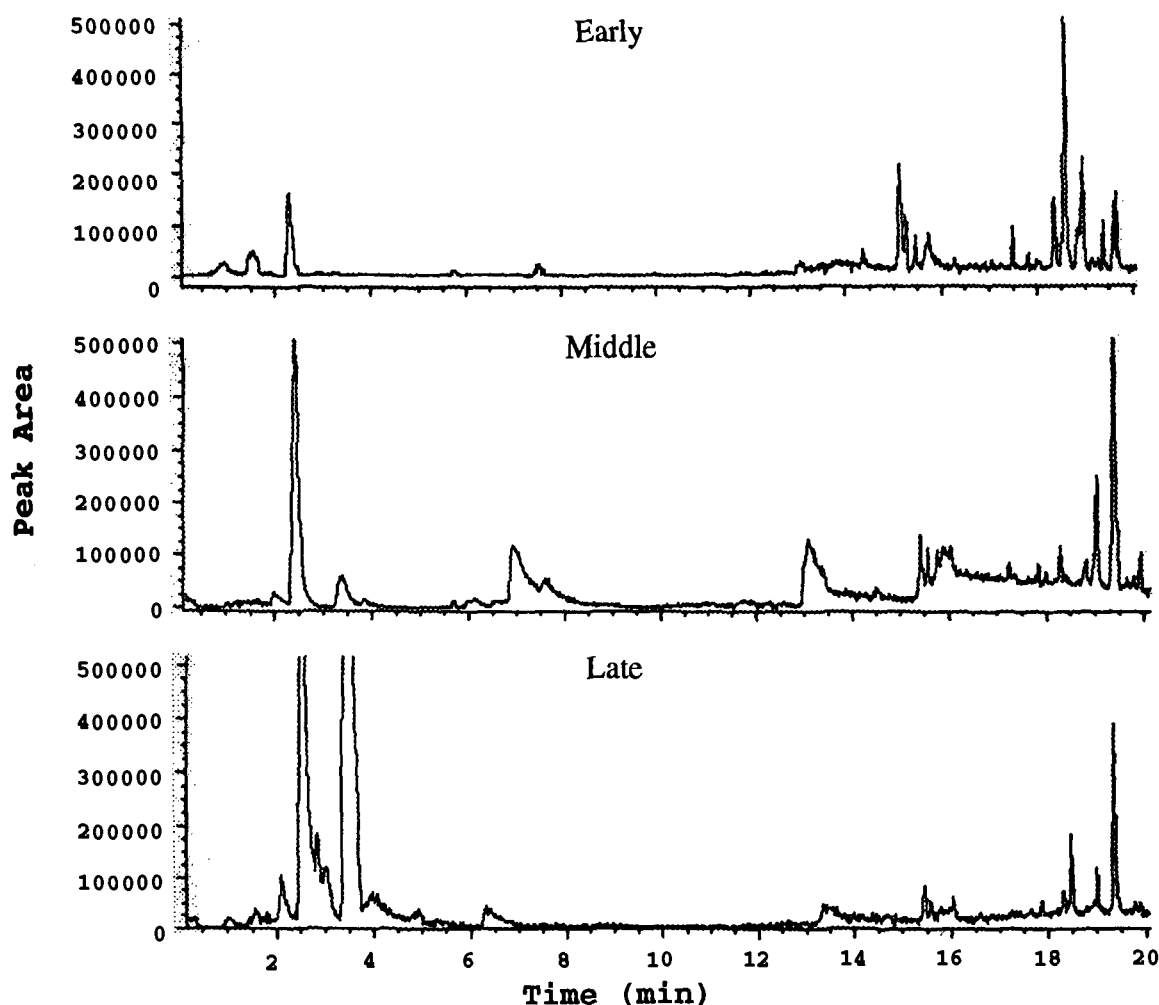


Fig. 5. Representative chromatograms of volatile organic compounds in headspace atmosphere of Treatment 4 (alfalfa + streptomycin) at early, middle, and late stages of the experiment.

iment. The temporal patterns of VOC production in Treatments 2, 3, and 4 were similar in that compounds with longer retention times, >16 min (those compounds that took longer to pass through the column of the gas chromatograph), were detected earlier in the experiment and then decreased as two compounds with shorter retention times (≈ 2.5 and 3.5 min) became abundant and then decreased with time (Fig. 5). In the headspace atmosphere of Treatment 5, compounds with longer retention times (>16 min) were detected early in the experiment along with compounds with retention times between 12 and 16 min and one broad peak between 7 and 8 min (Fig. 6). As most of the longer retention time peaks quickly disappeared, production of the other groups increased and a compound with a retention time of ≈ 2.5 min appeared. Production of all VOCs then slowly declined with time.

Thirty-two different VOCs were detected from the soil microcosms monitored in this study. Only nine of these compounds could be identified by matching to entries in the Wylie Library of Chemical Compounds (matching quality values between 50 to 99, on a scale of 0 to 100). Of the 32 compounds detected, 19 were observed in only one treatment, six were observed in two treatments,

and only four were detected in all five treatments. Of the 19 VOCs observed in only one treatment, 13 were produced in the microcosms dominated by bacteria and actinomycetes (Treatment 5).

Geosmin (retention time ≈ 18.5 min) was occasionally detected in small amounts only in microcosms that had substantial activity of all three groups of microorganisms (Treatment 2). Although compounds with retention times of ≈ 18.5 min were also detected in Treatments 4 and 5 (Fig. 4, 5, and 6), mass spectral analysis indicated that they were not geosmin or any other compound listed in the Wylie database. 2-Methylisoborneol (retention time ≈ 14.6 min) was detected only in the treatment dominated by actinomycetes and bacteria (Treatment 5) but was observed regularly and in significant amounts. A compound identified as cyclohepten-1-one (retention time ≈ 2.5 min) was detected in all treatments. Treatments 2 and 5 produced a compound identified as 4-ethylidene cyclohexene (retention time ≈ 3.5 min). In the microcosms dominated by fungal growth, three of the four compounds matched in the Wylie database were identified as terpenes (retention times between 16 and 18 min).

All treatments, with the exception of Treatment 4,

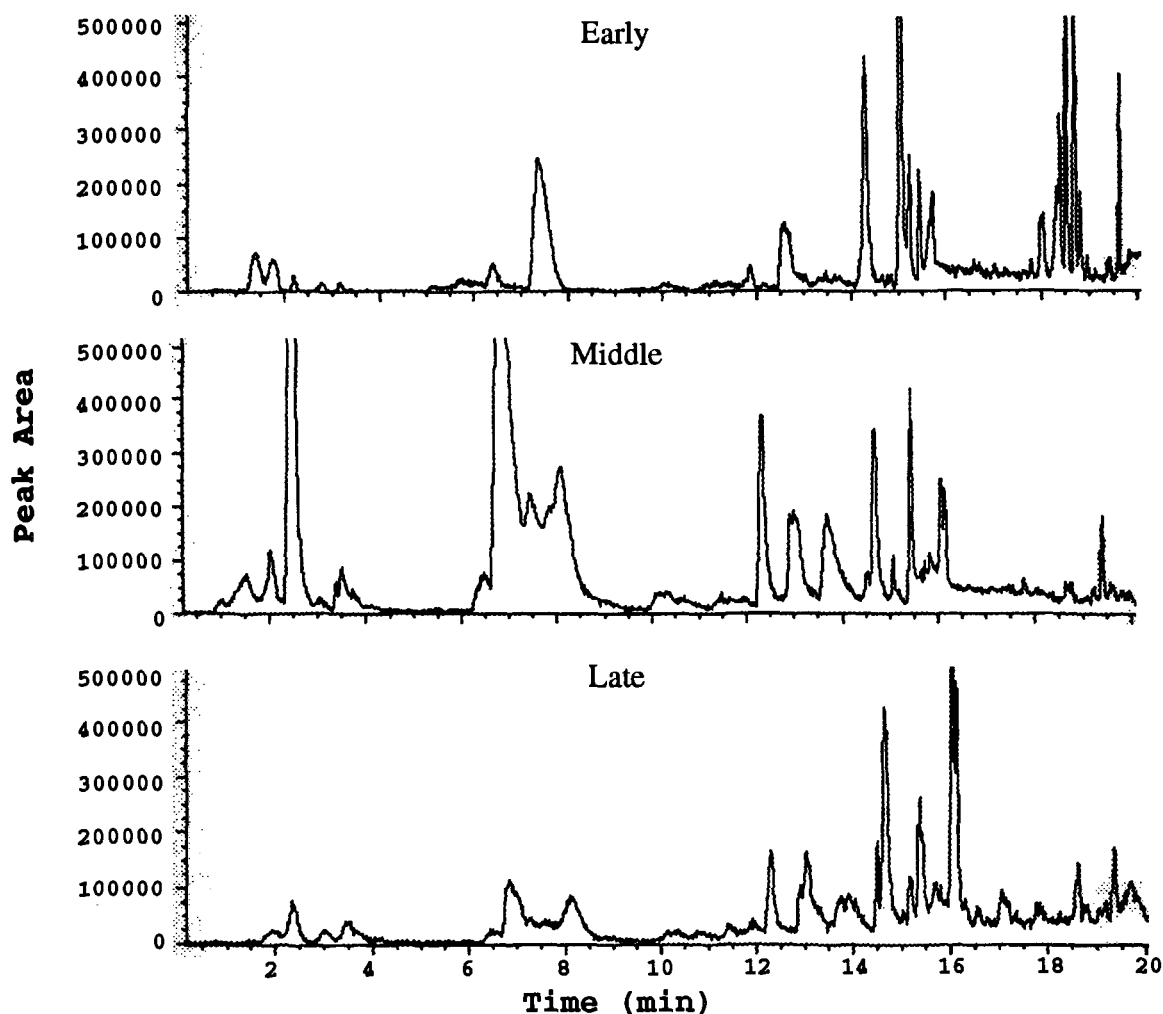


Fig. 6. Representative chromatograms of volatile organic compounds in headspace atmosphere of Treatment 5 (alfalfa + cycloheximide) at early, middle, and late stages of the experiment.

had earthy, musty odors that differed in intensity as determined by smell. The strongest odor was in microcosms dominated by actinomycetes and bacteria (Treatment 5) followed by the microcosms with substantial activity of all three groups (Treatment 2). The odor from microcosms dominated by fungi (Treatment 4) was characterized as sour.

DISCUSSION

Results obtained in this experiment support our hypothesis that soils populated by different microbial communities will emit different kinds and amounts of VOCs. In our experiment, treatments with low microbial activity, as indicated by CO_2 evolution and direct microscopic counts, had relatively low VOC production while those with higher microbial activity produced much greater amounts of VOCs (Fig. 2, 3, and 4). Treatments dominated by different groups of microorganisms (actinomycetes, bacteria, or fungi) produced different kinds of VOCs (Table 1, Fig. 4). Chromatograms of microcosm headspace atmospheres provided fingerprints or profiles of VOC production in all treatments for comparison. The chromatograms for each treatment were different at

each sampling date and also changed with time within a given treatment (Fig. 4, 5, and 6). For example, in Treatment 4 (Fig. 5) several terpenes, identified as camphene, naphthalene, and aromadendrene, were detected in the first 2 wk of the experiment and then became less common as the production of two other compounds, identified as cyclohepten-1-one and 4-ethylidene cyclohexene, increased and then declined. In Treatment 5 (Fig. 6), dominated by actinomycetes and bacteria, several compounds with retention times between 12 and 18 min (including 2-methylisoborneol but no terpenes) were also detected in the first 2 wk. As production of these compounds decreased, an unidentified compound (retention time ≈ 7.5 min.) along with cyclohepten-1-one and 4-ethylidene cyclohexene appeared and then declined.

2-Methylisoborneol was detected only in Treatment 5, which had the greatest amount of actinomycetal and bacterial growth, suggesting that these organisms are primarily responsible for the production of this compound in soil. On the other hand, no geosmin was detected in this same treatment. Unpublished data from our laboratory indicate that 2-methylisoborneol is one of the most abundantly produced VOCs by soil-isolated actinomycetes grown on agar in pure culture. Geosmin was detected

Table 1. Production of volatile organic compounds (VOCs) in soil microcosms throughout the course of the experiment.

Treatment	Number of different VOCs detected	VOCs identified
1 (control)	4	Cyclohepten-1-one
2 (alfalfa)	12	Camphene Cyclohepten-1-one <i>Geosmin</i> [†] 4-ethylidene cyclohexene 2-methyl-2-bornene
3 (straw)	6	Cyclohepten-1-one
4 (alfalfa + streptomycin)	15	Camphene <i>Naphthalene</i> Cyclohepten-1-one <i>Aromadendrene</i>
5 (alfalfa + cycloheximide)	22	2-methyl-2-bornene <i>2-methylisoborneol</i> 4-ethylidene cyclohexene Cyclohepten-1-one

[†] Compounds in italics were found in one treatment only.

only in microcosms in which there was substantial activity of all three groups of microorganisms (Treatment 2), making it difficult to determine its source because it was not detected in any of the treatments in which the microbial community was dominated by a particular group. Additionally, results of a previous study from our lab (Stahl and Parkin, 1994) indicate that geosmin may be strongly sorbed to surfaces in soil. This suggests that geosmin production may be more common than our results indicate but because this compound does not readily escape from soil, it may, at times, be absent from the headspace atmosphere.

Mass spectral analyses of compounds collected from Treatment 4, dominated by fungi, indicate that terpenes were the type of VOC produced in greatest quantity by these organisms in our study. The particular compound produced in largest amounts in Treatment 4 was matched to camphene in the Wylie database. Two additional compounds commonly produced in microcosms dominated by fungi were matched to naphthalene and aromadendrene while a number of other VOCs collected were matched to other terpenes in the database. In a number of other studies, terpenes have been found to be one of the most abundantly produced groups of volatiles by fungi growing on agar and cereal grains (Borjesson et al., 1990, 1992) and naphthalene has previously been identified as a volatile metabolite of certain fungi (Karahadian et al., 1985). In an examination of VOCs emitted from forest floors, Hanson and Hoffman (1994) found that terpenes comprised virtually all of the non-methane organic compounds escaping from these soils. The soil cores examined by Hanson and Hoffman, however, contained plant roots that probably contributed to the production of VOCs. Terpenes are among the most frequently reported volatiles from plants so it is likely that these compounds in soil are of both plant and fungal origin.

The high number of different VOCs detected in Treatment 5 suggests that actinomycetes and bacteria have the potential to produce more different kinds of VOCs in soil than do fungi. Only one compound produced in Treatment 5 was matched to a substance in the Wylie database, 4-ethylidene cyclohexene. Because of the lack of information on the VOCs produced in Treatment 5,

little can be said as to their nature; however, none of these compounds were matched to terpenes in the Wylie database.

The total amounts of VOCs produced in Treatments 4 and 5 were more than double the amount produced in Treatment 2; however, differences in microbial biomass, as indicated by direct microscopic counts, were not nearly as great. This observation suggests that the disturbance, or effect, created by antibiotics in soil resulted in greater microbial production of VOCs. Another possible explanation is that there is more concurrent microbial degradation of the VOCs produced in Treatment 2 because no antibiotics were added.

Although peak VOC production always occurred 6 to 12 d after the highest level of headspace CO₂ concentration in all treatments, there appeared to be little correlation between VOC and CO₂ production. Highest headspace CO₂ concentrations were observed in Treatment 4, yet VOC production was much greater in Treatment 5 (Fig. 3). This indicates that VOCs and CO₂ are indicators of different kinds of activity. A lack of correlation between VOC and CO₂ production was also reported by Borjesson et al. (1992) in their studies of fungi growing on cereal grains.

In conclusion, the results of this experiment indicate that soils with different microbial communities will produce different kinds and amounts of VOCs. Relating the nature of the microbial community to soil VOC emissions is complicated, however, because VOCs present in natural soils may be derived from a variety of sources, both internal and external to the soil. Types of substrates and metabolic condition of soil microorganisms also play an important role in determining the VOCs produced (Stotzky and Schenck, 1976). Our data, from this and other studies, indicate that the earthy/musty odor of soil cannot be attributed solely to geosmin production by actinomycetes, but instead, there are numerous VOCs produced by different microorganisms that contribute to the unique odor of soil. The results of this study support the hypothesis that 2-methylisoborneol may be a good indicator of metabolic activity of actinomycetes or actinomycetes and bacteria in soil; however, the use of geosmin as an indicator of microbial activity in soil was not supported. Terpenes appear to be the VOCs most abundantly produced by fungi, but their usefulness as indicators of fungal activity in soil may be limited by the fact that they are also commonly produced by plant roots.

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